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(71) Applicant: CALBIOCHEM NOVABIOCHEM CORPORATION [US/US]; 10394 Pacific Center Court, San Diego, CA 92121 (US).

(72) Inventors: JOYCE, Alison; 24 Rollins Street, Groveland, MA 01834 (US). WINCHELL, Lisa, F.; 447 Chapman Street, Canton, MA 02031 (US). DEMEO, Laura; 25 Boston Avenue, Somerville, MA 02144 (US). PROVUNCHER, Gail; 1 Dena Drive, Franklin, MA 02038 (US). MILLER, Thomas, E.; 246 Leyden Street, East Boston, MA 02128 (US). SORENSEN, Craig, M.; 131 Sumner Road, Boston, MA 02446 (US). HILL, David, E.; 85 Ridge Street, Arlington, MA 02474 (US).

(74) Agent: EISENSTEIN, Ronald, I.; Nixon Peabody LLP, 101 Federal Street, Boston, MA 02110 (US).

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(57) Abstract

The present invention employs an improved immunoassay that detects all forms of MMP-9 (both the form complexed with TIMP and the "free" uncomplexed form) in biological samples including human serum and plasma. Using this immunoassay the present inventors have surprisingly discovered that a much higher degree of detection and quantification is possible than with prior assays. The assay is preferably a capture assay using two antibodies to MMP-9 that do not compete with each other (i.e., bind to distinct epitopes). The epitopes that are bound to must be exposed in both the complexed MMP-9 as well as the free MMP-9.

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DIAGNOSTIC ASSAY FOR MATRIX METALLOPROTEINASE 9 AND USE THEREOF

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to assay methods for quantitation of metalloproteinase 9 (MMP-9) in biological samples using immunocapture techniques. The assay is designed to quantify both free MMP-9 and MMP-9 complexed with the tissue inhibitor of metalloproteinase 9, TIMP-1. In one preferred embodiment, this invention relates to methods and techniques for specifically selecting and isolating an MMP-9 from a biological sample, followed by the measurement of the amount of MMP-9, by using an antibody. The assay method is useful in the diagnosis and prognosis of diseases and disorders relating to MMP-9, including cancer.

2. Background

The interactions of cells with the extracellular matrix (ECM) are critical for the normal development and function of the organism. Modulation of cell-matrix interactions occurs through the action of unique proteolytic systems responsible for hydrolysis of a variety of ECM components. By regulating the integrity and composition of the ECM structure, these enzyme systems play a pivotal role in the control of signals elicited by matrix molecules, which regulate cell proliferation, differentation and cell death. The turnover and remodeling of ECM must be highly regulated since uncontrolled proteolysis contributes to abnormal development and to the generation of many pathological conditions characterized by either excessive degradation or lack of degradation of ECM components. See, Massova et al., *FASEB J.* 12:1075-1095 (1998).

An accumulating body of evidence suggests that the remodeling of ECM that occurs during normal growth, wound repair and angiogenesis as well as during the development and progression of pathologic conditions including malignant diseases, is accomplished largely through the action of MMPs. Birkedal-Hansen, H., *Current Opinions in Cell Bio.*

7:728-735, 1995; Matrisian, L. *Trends Genet.* 6:121-125, 1990; Woessner, J.F. *Ann. N.Y. Acad. Sci.* 732:11-21, 1994.

The MMPs are members of a multigene family of metal-dependent enzymes. These proteases have been classified into four broad categories originally based on substrate specificity. These specific enzymes are the collagenases (MMP-1/EC3.4.24.7; MMP-8/EC3.4.24.34;MMP-13), the gelatinases A(MMP-2/EC3.4.24.24) and B(MMP-9/EC3.4.24.35), the stromelysins (MMP-3/EC3.4.24.17:MMP-10/EC3.4.24.22; MMP-11/EC3.4.24.7) including a metalloelastase (MMP-12) and the membrane MMPs (MMP-14). Birkedal-Hansen, H., *Current Opinions in Cell Bio.* 7:728-735, 1995; Matrisian, L. *Trends Genet.* 6:121-125, 1990; Woessner, J.F. *Ann. N.Y. Acad. Sci.* 732:11-21, 1994. It is anticipated that additional MMPs with novel substrate specificity will be identified.

The regulation of MMP activity occurs at several levels including gene transcriptional control, proenzyme activation and inhibition of activated MMPs by endogenous inhibitors. Like many other enzyme families, the MMPs are a key component of a system of "balanced proteolysis" wherein a finely tuned equilibrium exists between the amount of active enzyme and its proteinase inhibitor(s). Liotta, et al. *Cell.* 64:327-336, 1991. These native metalloproteinase inhibitors comprise a family of proteins generally referred to as the TIMPS (Tissue Inhibitor of MetalloProteinase). Docherty, et al. *Nature.* 318:66-69, 1985; Carmichael, et al. *Proc. Natl. Acad. Sci. USA.* 83:2407-2411, 1986; Moses, et al. *J. Cell. Biochem.* 47:230-235,1991; Murray, et al. *J. Biol. Chem.* 261:4154-4159, 1986; Stetler-Stevenson, et al. *J. Biol. Chem.* 29:17374-17378, 1989; Pavloff, et al. *J. Biol. Chem.* 267:17321-17326, 1992; DeClerk, et al. *J. Biol. Chem.* 264:17445-17453, 1989.

Matrix metalloproteinase-9 (MMP-9; 92-kDa gelatinase B) is an important member of the MMP family involved in tumor invasion and metastasis. Woodhouse, et al. *Cancer.* 80 (Suppl. 8):1529-1537, 1997; Chambers, et al. *J. Natl. Cancer Inst.* 89:1260-1270, 1997; Coussens, et al. *Chem. Biol.* 3:895-904, 1996; Yu, et al. *Drugs Aging.* 11:229-244, 1997; Kohn, et al. *Cancer Res.* 55:1856-1862, 1995. MMP-9 is a collagenase with specificity for type IV collagen, which makes up the backbone of the basement

membrane. The proteolytic degradation of the ECM is an important aspect of many physiological and pathological conditions associated with alterations in connective tissue proteins such as embryo implantation, morphogenesis, wound healing, ovulation, cell migration, tissue involution, angiogenesis, and tumor invasion. DeClerk, et al. *Adv. Exp. Med. Biol.* 425:89-97, 1997. MMP-9 is secreted from stimulated macrophages, neutrophils and transformed cells in a latent form. In fact all MMPs, except membrane-type metalloproteinases, are secreted as inactive zymogens into the extracellular matrix, where subsequent activation results in cleavage of the proenzymes into the active species. The presence or absence of activators and the binding of tissue inhibitors of metalloproteinases (TIMPs) maintains strict control on the activation of such enzymes in the extracellular space. Borden, et al. *Crit. Rev. Eukaryot. Gen. Expression.* 7:159-178, 1997; Gomis-Ruth, et al. *Nature* (London). *389:77-81, 1997.* The TIMP family has four members: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Borden, et al. *Crit. Rev. Eukaryot. Gen. Expression.* 7:159-178, 1997; Fridman, et al. *Biochem J.* 289:411-416, 1993. Binding of the TIMPs to their specific MMPs regulates processing and activation of enzymatic activity of MMPs. In the case of gelatinases (MMP-9 and MMP-2), the TIMPs have been shown to bind to the zymogen forms of the enzymes. Fridman, et al. *Biochem J.* 289:411-416, 1993; Goldberg, et al. *J. Biol. Chem.* 267:4583-4591, 1992.

The MMP-9 protein has four structural domains: an amino-terminal propeptide, a catalytic domain, a fibronectin type-II like domain within the catalytic domain and a hemopexin-like domain at the carboxyl-terminal. Massova, et al. *FASEB J.* 12:1075-1095, 1998. Cleavage of the propeptide results in zymogen activation. The catalytic domain contains two zinc ions and a calcium ion. MMP-9 incorporates three repeats homologous to the type-II module of fibronectin into the catalytic domain. This region is also known as the gelatin binding domain and is involved in binding to denatured collagen or gelatin. Murphy, et al. *J. Biol. Chem.* 269:6632-6636, 1994. The hemopexin-like domain is highly conserved among MMPs and shows sequence similarity to the plasma protein, hemopexin. The hemopexin like domain has been shown to play a functional role in substrate binding and/or in interactions with the tissue inhibitors of metalloproteinases (TIMPs). There is a high degree of specificity in the

binding of TIMPs to the latent forms of these enzymes. TIMP-1 binds exclusively to the zymogen form of MMP-9 (Kd~35 nM), whereas TIMP-2 binds to the zymogen form of MMP-2 (Kd ~5 nM). Murphy, et al. *Matrix Biol.* 15:511-518, 1997. There is a biphasic binding of TIMP-1 to the zymogen form of MMP-9, with the hemopexin-like domain representing the high affinity binding site. Olson, et al. *J. Biol. Chem.* 272:29975-29983, 1997.

Traditional immunoassays detect mainly uncomplexed pro-MMP-9 and thus have poor clinical utility in that only a small percent of cancer samples read above normal samples.

As MMPs play an important role in normal and pathological process, including embryogenesis, wound healing, inflammation, arthritis and cancer, it would be desirable to have a highly specific and accurate assay to measure MMP levels in patient samples.

SUMMARY OF THE INVENTION

The present invention employs an improved immunoassay that detects all forms of MMP-9 (both the form complexed with TIMP and the "free" uncomplexed form) in biological samples including human serum and plasma. Using this immunoassay the present inventors have surprisingly discovered that a much higher degree of detection and quanitification is possible than with prior assays. The assay is preferably a capture assay using two antibodies to MMP-9 that do not compete with each other (i.e., bind to distinct epitopes). The epitopes that are bound to must be exposed in both the complexed MMP-9 as well as the free MMP-9.

In one preferred embodiment, we found that one can use a detector MMP-9 antibody and a capture anti- MMP-9 antibody. For example, serum pro-matrix metalloproteinase-9 (precursor of matrix metalloproteinase 9, pro-MMP-9, progelatinase B) and pro-MMP-9/TIMP1 complex levels [pro-MMP-9 noncovalently bound to tissue inhibitor of metalloproteinases (TIMP-1)] are elevated in cancer patients. These levels can be

determined by using a monoclonal anti-MMP-9 antibody such as clone 6-6B (ORP product MMP9 (Ab-1), Cat #IMO9L) and a polyclonal antibody to MMP-9 which is available from BioDesign (Cat.# K90163C). Unlike the traditional immunoassay, the improved immunoassay of the present invention has excellent clinical utility. For example, ninety percent of cancer sera read above normal samples and most of the pro-MMP-9 in cancer sera is found to be complexed with TIMP-1.

The present invention provides a method of determining the presence of an MMP-9 associated disorder, e.g., cancer, comprising the step of assaying a biological sample obtained from a patient for the presence of free MMP and MMP/TIMP complexes, such as MMP-9 and pro-MMP-9/TIMP-1 complexes. The presence of MMP-9 above normal limits correlates with the presence of cancer. The measurement of the level of MMP-9 in a biological sample is useful in the diagnosis, prognosis, and treatment of cancer as well as monitoring of a patients response to therapy and staging of disease.

Using this assay, the present inventors have detected pro-MMP-9 protein complexed with TIMP-1 and uncomplexed MMP-9 in human sera and plasma samples and cell culture supernatants. Specificity was demonstrated by immunoaffinity extraction (inhibition of assay signal) of pro-MMP-9 positive samples by a specific pro-MMP-9 antibody and a specific TIMP-1 antibody. For instance, an MMP-9 antibody, which is not a component of the ELISA, extracted the MMP-9 which leads to loss of signal in the assay (almost all the signal was lost), while the control antibody (e.g., a non MMP-9) did not affect the signal of the MMP-9 positive samples (see Figure 3). A TIMP-1 antibody extracted pro-MMP-9 activity detected by this immunoassay, but very little of the pro-MMP-9 activity detected by other traditional immunoassays which shows that the traditional assay detects mainly uncomplexed MMP-9 protein and therefore fails to accurately measure MMP-9 levels in patient samples. Addition of TIMP-1 and TIMP-2 recombinant proteins to both positive and negative samples does not change the level of MMP-9 detected.

Thus, the present invention further provides a general method for the detection and

quantitation of a MMP-9 in a biological fluid from a human. The method comprising the steps of:

(a) exposing an amount of biological sample to a first antibody which specifically binds an epitope of MMP-9 exposed in either the free or complexed form in solution phase or adsorbed onto the solid support so that any MMP-9 in the biological sample is captured by the antibody;

(b) exposing the captured MMP-9 to a second detectably labeled antibody which specifically binds a second epitope that is exposed in either the free or compound form, preferably a pro-MMP-9;

(c) removing unbound material; and

(d) detecting and quantifying an amount of the second detectably labeled antibody detected.

In respect to the first and second antibodies, preferably one antibody is polyclonal and the other is monoclonal.

The amount of MMP-9 in the biological sample is determined from the amount of detectably labeled antibody bound to the solid support. The detectably labeled antibodies can be labeled with a detectable substance such as, for example, biotin, radioactive agents, chromophoric agents, enzymatic agents, chemiluminescent, fluorometric and haptenic.

The method of the invention for measuring MMP levels in biological samples contemplates the use of a capture antibody, which can be a monoclonal, polyclonal recombinant or chimeric antibody, and a detectably labeled antibody, which can also be a monoclonal, polyclonal recombinant or chimeric antibody.

The present invention further provides a kit useful for the detection of MMP-9 in a

biological sample. This kit comprises antibodies which specifically bind MMP-9 to capture the MMP-9 present in the sample and a detectably labeled antibody which specifically binds MMP-9, such as pro-MMP-9, to quantify the amount of MMP-9.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a standard curve: The mean signal of of each standard run in replicates of four in eight assays using two different lots of plates and two lots of detector antibody.

Figure 2 shows the sensitivity of one embodiment of the assay of the present invention: The lower limit of detection (LLD), commonly used to define sensitivity, was measured by assaying four replicates of zero eight times using two different lots of plates and two different lots of detector antibody. The grand mean signal and pooled standard deviation of zero was calculated. The grand mean of each standard (run in replicates of four in the eight assays) was used for the standard curve (Figure 1), and the response, mean signal of zero plus two standard deviations, read in dose from the standard curve is the LLD; that is, the smallest dose that is not zero with 95% confidence.

Figure 3 shows the specificity of an assay of the present invention: Levels of MMP-9 detected by the ELISA after immunoaffinity extraction of MMP-9 positive samples (PMA treated HT1080 and HL-60 cells) with a MMP-9 antibody that is not used in the ELISA, a TIMP-1 antibody, a TIMP-2 antibody and a TIMP-3 antibody.

Figures 4A-4D show levels of MMP-9 detected by the MMP-9 ELISA of the present invention after 2-aminophenylmercuric acetate (APMA) treatment. APMA promotes the autocatalytic cleavage of the N-terminal prosequence of the latent 92-kDa enzyme to yield the active form of the enzyme. Shapiro, et al. *J. Biol. Chem.* 270: 6351-6356, 1995. Analysis of the samples by zymography showed both cleavage of the proenzyme by APMA and a good correlation with the levels of the proenzyme determined with the

MMP-9 ELISA. Several methods of MMP-9 induction were used to generate positive samples such as treatment with 50 ng/ml amphiregulin (AR), 50 ng/ml epidermal growth factor (EGF), 1ng/ml transforming growth factor beta (TGFb) and 25 ng/ml phorbol 12-myristate 13-acetate (PMA). Normal human sera (NHS) containing moderate levels of MMP-9 were as treated with APMA and analyzed.

Figure 5 shows phorbol ester induced up-regulation of MMP-9 protein in HL-60 cells. It has been shown that the induction of MMP-9 gene expression is associated with macrophage differentiation. Xie, et al. *J. Biol. Chem.* 273:11576-11582, 1998. Phorbol 12-myristate 13-acetate (PMA) at all concentrations studied induced the synthesis of MMP-9 protein. This up regulation closely parallels the timing of PMA induced cell adhesion and spreading, a hallmark of macrophage differentiation.

Figure 6 shows phorbol ester induced up-regulation of MMP-9 in HT1080. PMA has been shown to increase the expression of MMP-9 by HT1080 cells. Morodomi, et al. *Biochem. J.* 285: 603-611, 1992; Lim, et al. *J. Cell. Physiol.* 167:333-340, 1996. The MMP-9 ELISA of the present invention detects this increase in synthesis in a time dependent matter.

Figures 7A and 7B show detection of MMP-9 in Human Sera and Plasma: Levels of MMP-9 detected by the assay of the present invention are significantly elevated in cancer sera samples compared to normal sera and plasma samples (left figure is the data on a logarithmic scale and the right is a linear scale). The following samples were assayed: normal human sera (NHS), normal human plasma (NHP), sera from cervical cancer (CV), breast cancer (BR) and prostate cancer (PT) patients.

Figure 8 shows the clinical utility of the MMP-9 Immunoassay of the present invention. The assay has a 90% percent clinical sensitivity (ability to recognize affected individuals) at a 95 % clinical specificity (ability to recognize unaffected individuals).

Figure 9 shows parallelism using the assay of the present invention: The study tested dilution-recovery of ten positive samples. The dilutions were run in the MMP-9

ELISA and the "found" doses were plotted against the "expected" doses to determine linearity of dilution. The slope is not significantly different from one and the intercept is not significantly different from zero. These studies are consistent with the absence of cross-reacting and matrix effects such as pH, salts, and endogenous binders that interfere with the reagents used in the assay. Note: Ca = Cancer; NHS = Normal Human Sera; NHP = Normal Human Plasma; PMA = Phorbol 12-myristate 13-acetate; EGF = Epidermal Growth Factor; AR = Amphiregulin; Both = EGF and AR; A = 2×10^6 cells/ml; B = 1.0×10^6 cells/ml.

Figure 10 shows the precision of the assay of the present invention: The pooled coefficients of variation (according to the formula of Henry, R.J., Cannon, C.D., and Winkleman, J.W., *Clinical Chemistry*, 1974 Harper and Row, New York, N.Y.) and between assay coefficients of variation are plotted against MMP-9 concentrations. The pooled data were collected from samples run eight times using two different lots of plates and two different lots of detector antibody in replicates of four on two separate occasions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an immunoassay, e.g., a "sandwich" enzyme immunoassay employing antibodies specific for both free and complexed MMP-9. The epitopes need to be exposed in both the free and complexed form. Preferably the detector antibody binds the pro-MMP-9 form. For example, in one embodiment of this assay the antibody, specific for the human MMP-9 protein, is immobilized onto surface of a solid phase support, e.g., an immunoassay plate, (other solid supports such as beads, tubes or magnetic particles can be used as well). The sample to be assayed along with a labeled, e.g., biotinylated, detector antibody that is specific for pro-MMP-9 is added. For example, the detector antibody can be simultaneously pipetted into the wells and allowed to incubate for a period of time (e.g., two hours), during which any MMP-9 present binds to both the capture and detecting antibodies. Unbound material is washed away and, if the detector antibody is biotinylated, horseradish peroxidase-conjugated streptavidin is added, which binds to the detector antibody. The horseradish peroxidase catalyzes the

conversion of a chromogenic substrate (i.e., tetramethylbenzidine) from a colorless solution to a colored solution, the intensity of which is proportional to the amount of pro-MMP-9 protein in the sample. The colored reaction product is quantified using a spectrophotometer.

Alternatively, other detection methods can be used in place of the streptavidin-biotin-horseradish peroxidase labeled immunologically responsive substance, such as radioactive agents, chemiluminescence agents, bioluminescence agents, fluorescence agents, or other chromophoric agents. In addition the detector antibody can be directly labeled or labeled indirectly with other molecules in place of biotin.

Quantitation is achieved by the construction of a standard curve using known concentrations of, e.g., the pro-MMP-9. By comparing the signal obtain from a sample containing an unknown amount of a pro-MMP-9 with that obtained from a known concentration of a pro-MMP-9, the concentration of the MMP-9 in the sample can be determined.

The preferred method of the present invention employs a standard ELISA assays known in the art. (See, Antibodies, A Laboratory Manual (Harlow, E. and Lane, D. eds.) 1988, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. and Immunology, A Practical Guide (Chan, E.W. et. al. eds) Academic Press Inc, San Diego, CA.) Preferably, an ELISA uses two antibodies that bind to nonoverlapping epitopes on the same antigen and may utilize either two antibodies that recognize discrete sites on an antigen or one batch of affinity-purified polyclonal antibodies. However, in other embodiments, the assay of the present invention can employ a capture antibody specific for both free and complexed MMP-9 and a non-antibody system for detecting the captured protein. For example, one could use a macromolecule that reacts with the captured MMP-9 or TIMP-1.

Antibodies which specifically bind MMP-9 can include human or animal (e.g., rabbit, mouse, rat, etc.) polyclonal or monoclonal or recombinant or chimeric antibodies. Methods used to prepare and purify polyclonal, monoclonal, recombinant and chimeric

antibodies are known in the art.

Such antibodies are also available commercially. Examples of some commercially available anti-MMP-9 antibodies include:

Antibodies:

Company	Antibody to	Type	
BioDesign	MMP-9	Sheep Polyclonal	
The Binding Site	MMP-9	Sheep-Polyclonal	
The Binding Site	MMP-9	Sheep-Polyclonal conjugated to peroxidase	
Fuji Chemical Industries	Bovine TIMP-1	Monoclonal	7-6C1
Fuji Chemical Industries	Human TIMP-1	Monoclonal	147-6D11
Fuji Chemical Industries	Human MMP-9	Monoclonal	56-2A4
Chemicon	MMP-9	Monoclonal	MAB1347
Chemicon	MMP-9	Rabbit polyclonal	AB805
Chemicon	MMP-9	Rabbit polyclonal	AB804
Chemicon	MMP-9	Rabbit polyclonal	AB16996
Chemicon	TIMP-1	Monoclonal	MAB1338
Chemicon	TIMP-1	Monoclonal	MAB1329
Chemicon	TIMP-1	Monoclonal	MAB1327
Chemicon	TIMP-1	Monoclonal	MAB1325
Chemicon	TIMP-1	Monoclonal	MAB1370
Chemicon	TIMP-1	Monoclonal	MAB1371
Chemicon	TIMP-1	Rabbit polyclonal	AB800
Chemicon	TIMP-1	Rabbit polyclonal	AB770
Chemicon	TIMP-1	Rabbit polyclonal	AB8112
Chemicon	TIMP-1	Rabbit polyclonal	AB8206
Oncogene Research Products	MMP-9	Monoclonal	IM09L
Oncogene Research Products	MMP-9	Monoclonal	IM10L
Oncogene Research Products	MMP-9	Monoclonal	IM37L
Santa Cruz Biotechnology	MMP-9	Goat Polyclonal	sc-6840
Santa Cruz Biotechnology	MMP-9	Goat Polyclonal	sc-6841

The detectably labeled antibody can be labeled using any of a variety of labels and methods of labeling known in the art. Examples of types of labels encompassed by the present invention include, but are not limited to, radioisotopic labels (e.g., ^{125}I, ^{131}I, ^{35}S, ^{14}C, etc.), non-radioactive isotopic labels ^{55}Mn, ^{56}Fe, etc.), fluorescent labels (e.g., a

fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, a fluorescamine label, etc.), chemiluminescent labels, enzyme labels (e.g., alkaline phosphatase, horse radish peroxidase, etc.), protein labels, etc.

Biological samples include body fluids, for example, plasma, serum, saliva, urine, lung lavage fluid, cyst fluid, etc., and tissue extracts wherein the tissue pieces are removed and separated from the liquid extract.

In the method of the present invention, the pro-MMP-9 present in a biological sample, for example, plasma, serum, saliva, urine, lung lavage fluid, cyst fluid, tissue extract, etc., is selectively captured from the sample by anti-pro-MMP-9 antibodies. The immunocapture assay can be performed in solution phase or on solid support as is well known in the art. In a preferred embodiment of the invention, the anti-pro-MMP-9 antibodies are affixed onto a solid support by any stable interaction known to the art such as hydrophobic, electrostatic or covalent interaction. Some examples of solid supports are microtiter plates, magnetic particles, beads, sheets, membranes, chromatography resins, e.g., Sepharose, etc. Preferably, multiwell microtiter plates or nitrocellulose membranes are used, so that when the pro-MMP-9 is captured by the antibodies, the resultant immunological complex is immobilized and easily isolated, e.g., by rinsing away non-binding material remaining in the biological sample.

The method of this invention has utility in screening biological samples to detect malignancy through the measurement of high levels of MMP9 and pro-MMP-9, in the sample material. This invention also contemplates the preparation of a kit useful for the detection of pro-MMP-9 in a biological sample. Such a kit comprises anti-pro-MMP-9 antibodies, adsorbed onto a solid support, preferably a microtiter plate having from approximately four to approximately ninety-six wells and a second anti-pro-MMP-9 antibody conjugated to a label; and, where necessary, a means for measuring said label.

It will be appreciated by those of ordinary skill in the art that the objects of this invention can be achieved without the expense of undue experimentation using well

known variants, modifications, or equivalents of the methods and techniques described herein. The skilled artisan will also appreciate that alternative means, other than those specifically described, are available in the art to achieve the method of MMP-9 measurement described herein and how to employ those alternatives to achieve functional equivalents of the method of the present invention. It is intended that the present invention include those variants, modifications, alternatives, and equivalents which are appreciated by the skilled artisan and encompassed by the spirit and scope of the present disclosure.

It is understood in the art that modifications may be made to the reaction conditions and specific elements used in the immunocapture enzyme assay to enhance the sensitivity of the assay to measure MMP. For example, it is contemplated that a substitution may be made in the choice of the chromogenic substrate or that a ligand (e.g., chemical group) may be added to the signal antibody without affecting the specificity of the assay of this invention. It will also be understood that optimization of e.g., MMP determination encompasses art-known alterations and modifications of reagents and conditions of the method of invention, as will be apparent to those skilled in the art using the teachings of this disclosure.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

The MMP-9 ELISA disclosed in this example is a "sandwich" enzyme immunoassay employing a mouse monoclonal antibody and a sheep polyclonal antibody. An antibody, specific for the human MMP-9 protein, has been immobilized onto the surface of microtiter wells using standard techniques. The sample to be assayed and biotinylated detector monoclonal antibody are pipetted into the wells and allowed to incubate for two hours, during which time any MMP-9 present binds to the capture and detecting

antibodies. Unbound material is washed away and horseradish peroxidase-conjugated streptavidin is added, which binds to the detector antibody.

The detector antibody is anti-MMP-9 mouse monoclonal antibody clone 6-6B (ORP product MMP9 (Ab-1), Cat # IM09L). The capture antibody is an anti-MMP-9 polyclonal raised in sheep and obtained from BioDesign of Kennebunk, Maine (Cat # K90163C).

The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent), the intensity of which is proportional to the amount of MMP-9 protein in the sample. The colored reaction product is quantified using a spectrophotometer.

Quantitation is achieved by the construction of a standard curve using known concentrations of MMP-9. By comparing the absorbance obtained from a sample containing an unknown amount of MMP-9 with that obtained from the standards, the concentration of MMP-9 in the sample can be determined.

EXAMPLE 2

It is known that 2-aminophenylmercuric acetate (AMPA) treatment promotes the autocatalytic cleavage of the N-terminal prosequence of the latent 92-kDa enzyme to yield an 84-kDa enzyme. Further treatment causes the autocatalytic cleavage of the carboxyl terminus to generate the 68-kDa form of MMP-9. Both tissue culture supernatants and sera samples were tested in the MMP-9 ELISA after APMA treatment. In every case almost all the signal disappeared after APMA treatment (see Figure 4). Analysis of the samples by zymography showed both cleavage of the proenzyme by APMA and a good correlation with the levels of the proenzyme determined with the MMP-9 ELISA. In addition the assay recognizes pure recombinant pro-MMP-9 protein, but not active MMP-9 recombinant protein. These results demonstrate that the assay of

the present invention specifically recognizes free and TIMP bound zymogen form of MMP-9.

EXAMPLE 3

This example discloses the use of one embodiment of the assay of the present invention.

Materials Provided

Standards should be assayed in duplicate. A standard curve must be performed on the same plate and at the same time as the samples. The MMP-9 ELISA provides sufficient reagents to run two sets of standard curves, and 41 samples (if assayed in duplicate all at once using one standard curve), or 34 samples (if assayed on two separate occasions using two standard curves).

- COATED MICROTITER PLATE: 96 removable wells coated with MMP-9 polyclonal antibody.
- MMP-9 STANDARD: two vials containing lyophilized MMP-9 protein calibrated to recombinant MMP-9 protein. Reconstituted standards should be discarded after one use.
- DETECTOR ANTIBODY: Biotinylated monoclonal anti-human MMP-9 antibody.
- 400X CONJUGATE: Streptavidin-Peroxidase Conjugate: 400-fold concentrated solution.
- CONJUGATE DILUENT: The buffer for dilution of 400X Conjugate.
- SUBSTRATE: The chromagenic substrate.
- SAMPLE DILUENT: A buffer used to dilute standards and samples.
- 20X PLATE WASH CONCENTRATE: 20-fold concentrated solution of PBS and surfactant. Contains 2% chloroacetamide.
- STOP SOLUTION: 2.5N sulfuric acid
- PLATE SEALERS: To cover plates during incubations.

Sample Preparation

For suspension cells: Pellet by centrifugation (1000 x g for 10 minutes, 4 °C) and remove supernatant for testing. Samples may be stored at –20 °C.

For adherent cells: Remove tissue culture media, centrifuge tissue culture media (1000 x g for 10 minutes), and remove supernatant for testing. Samples may be store at –20 °C.

Samples found to contain greater than 20 ng/ml MMP-9 (i.e., outside the range of the standard curve) must be diluted with Sample Diluent (provided), so that the MMP-9 concentration falls within the range spanned by the standard curve, and assayed again.

Detailed Protocol

The MMP-9 ELISA is provided with removable strips of wells so the assay can be carried out on two separate occasions. Since conditions may vary, a standard curve MUST be determined each time the assay is performed. Standards should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the empty well holder. Return any unused wells to the foil pouch, reseal and store at 4°C.
2. Prepare a working solution (1X) of Wash Buffer by adding 25 ml of the 20X concentrated solution (provided), to 475 ml of deionized water. Mix well.
3. Each time an assay is performed, reconstitute a Lyophilized Standard by carefully and accurately pipetting dH_2O and sample diluent, as described on the lyophilized MMP-9 Standard vial label to give a concentration of 20 ng/ml. Let the reconstituted standard sit for 15 minutes at room temperature, with occasional swirling. Avoid excessive agitation of the standard. After reconstituting the MMP-9 Standard it should be diluted with Sample Diluent. Obtain seven tubes and label them 20, 10, 5, 2.5, 1.25, 0.625 and 0 ng/ml. Add 250 µl of Sample Diluent into each tube except the 20ng/ml tube (first tube) which gets "undiluted" reconstituted standard. Remove 500 µl from the original vial of lyophilized material and add it to the first tube. Remove 250µl from the first tube (20 ng/ml) and add it to the second tube (10 ng/ml) and mix gently. Repeat this procedure until you reach the sixth tube (0.625 ng/ml).

The last tube (0 ng/ml) should just be Sample Diluent. Reconstituted standards should be discarded after one use.

4. Prepare all samples (see page 4). Sera and plasma samples should be diluted with Sample Diluent 1:10 (normal samples) or 1:40 (most cancer samples) or greater as needed.
5. Pipette 50 µl of the Detector Antibody into each well.
6. Add samples and each of the MMP-9 standards (in duplicate) by pipetting 50 µl into appropriate wells using clean pipette tips for each sample.
7. Cover wells with a plate sealer and incubate at room temperature for 2 hours.
8. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
9. Dilute a sufficient amount of the 400X Conjugate 1:400 in Conjugate Diluent to provide 100 µl of 1X solution for each sample and standard well (For example: add 30 µl to 12 ml of Conjugate Diluent), mix gently. Filter with a 0.2 µm syringe filter prior to use.
10. Pipette 100 µl of the 1X Conjugate into each well, cover with a plate sealer and incubate at room temperature for 30 minutes. Discard any unused 1X Conjugate.
11. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
12. FLOOD ENTIRE PLATE WITH dH_2O. Remove contents of wells by inverting over sink and tapping on paper towels.
13. Add 100 µl of Substrate Solution to each well and incubate IN THE DARK at room temperature for 30 minutes.
14. Add 100 µl of Stop Solution to each well in the same order as the previously added Substrate Solution.
15. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/595 nm (or 450/540nm). Wells must be read within 30 minutes of adding the Stop Solution.

Evaluation of Results

1. Average the duplicate absorbance values for each standard, including the zero, and all sample values.
2. On graph paper, plot the mean absorbance values for each of the standards on the Y axis, versus the concentration of each standard (ng/ml) on the X axis.
3. Determine the concentration of unknowns by interpolation from the standard curve. There are a variety of microtiter plate reader software packages available (Softmax, Molecular Devices Corporation, Menlo Park, CA; KinetiCalc, BioTek Instruments, Inc. Winooski, VT) for analysis of microtiter plate data, which simplifies this process.
4. For samples which have been diluted, the MMP-9 concentration must be multiplied by the dilution factor (ie., if the sample was diluted five-fold, then the MMP-9 value obtained from the standard curve must be multiplied by five).

The references cited in this application are herein incorporated by reference.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method for measuring free and complexed MMP-9 in a biological sample comprising the steps of:

(a) adsorbing an antibody which specifically binds to both the free and complexed form of MMP-9 onto a solid support;

(b) contacting an amount of said biological sample to said antibody adsorbed onto said support so that said MMP-9 is captured by said antibody adsorbed onto said solid support;

(c) contacting said captured MMP-9 to a detectably labeled antibody;

(d) rinsing away the non-binding material from said solid support;

(e) determining the amount of said label bound to said solid support.

2. The method of claim 1 wherein said biological sample is biological fluid, serum or plasma.

3. The method of claim 1 wherein said detectably labeled antibody is conjugated to a label selected from the group consisting of a radioisotope, a biotin, an aviden, a strep-aviden, a chromophore, a fluorophore, a chemiluminescent moiety, a hapten and an enzyme.

4. A kit useful for the measurement of a MMP-9 in a biological sample of a a human comprising:

a first antibody which specifically binds both the free and complexed form of MMP-9 absorbed onto a solid support; and

a second antibody which specifically binds MMP-9 conjugated to a label.

5. The kit of claim 4 wherein said label is selected from the group consisting of a radioisotope, a biotin, a chromophore, a fluorophore, a chemiluminescent moiety, a hapten and an enzyme.

6. A method for the detection of cancer in a patient comprising the steps of:

(a) obtaining from said patient a biological sample;

(b) contacting an amount of said biological sample to an antibody which specifically binds MMP-9 adsorbed onto a solid support so that said MMP-9 in said biological sample is captured by said antibody adsorbed onto said solid support;

(c) contacting said captured MMP-9 with a second antibody which specifically binds pro-MMP-9 conjugated to a label;

(d) rinsing away the non-binding material from said solid support;

(e) determining the amount of said label bound to said solid support; and

(f) whereby a condition of cancer is indicated for said patient exhibiting in step (e) a level of MMP-9 in said biological sample that is over and above the background or control normal level.

7. A method for measuring pro-MMP-9 comprising:

(a) adsorbing polyclonal antibodies which specifically bind to pro-MMP-9 onto a solid support;

(b) exposing an amount of human serum or plasma to the antibody bound to the solid

support such that the pro-MMP-9 in the serum or plasma is captured by the antibody bound to the solid support;

(c) exposing the captured pro-MMP-9 to a biotinylated monoclonal antibody which specifically binds MMP-9;

(d) rinsing away any material not bound to the solid support; and

(e) exposing the captured pro-MMP-9 to horseradish peroxidase- conjugated streptavidin and a chromogenic substrate such as tetramethylbenzidine to measure peroxidase activity as a measure of the amount of MMP-9 in the serum or plasma.

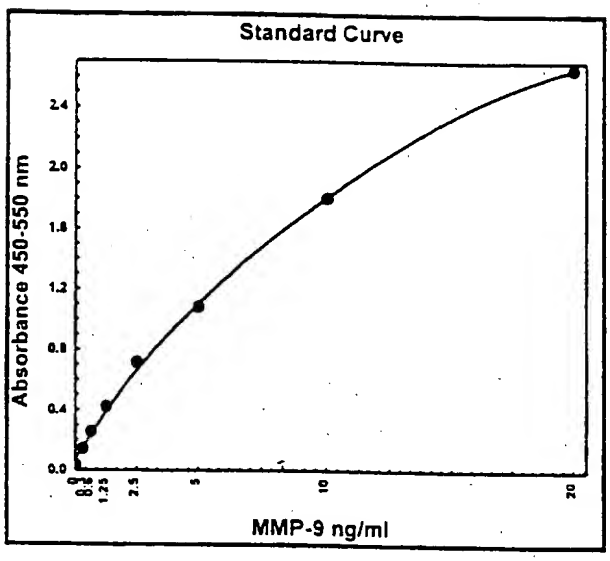


Figure 1.

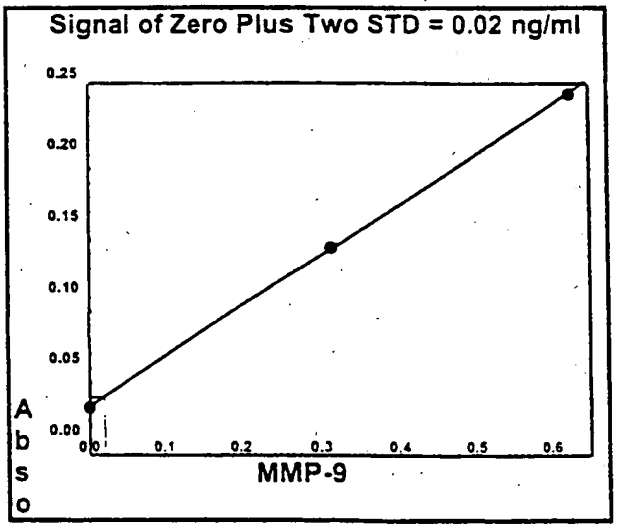


Figure 2.

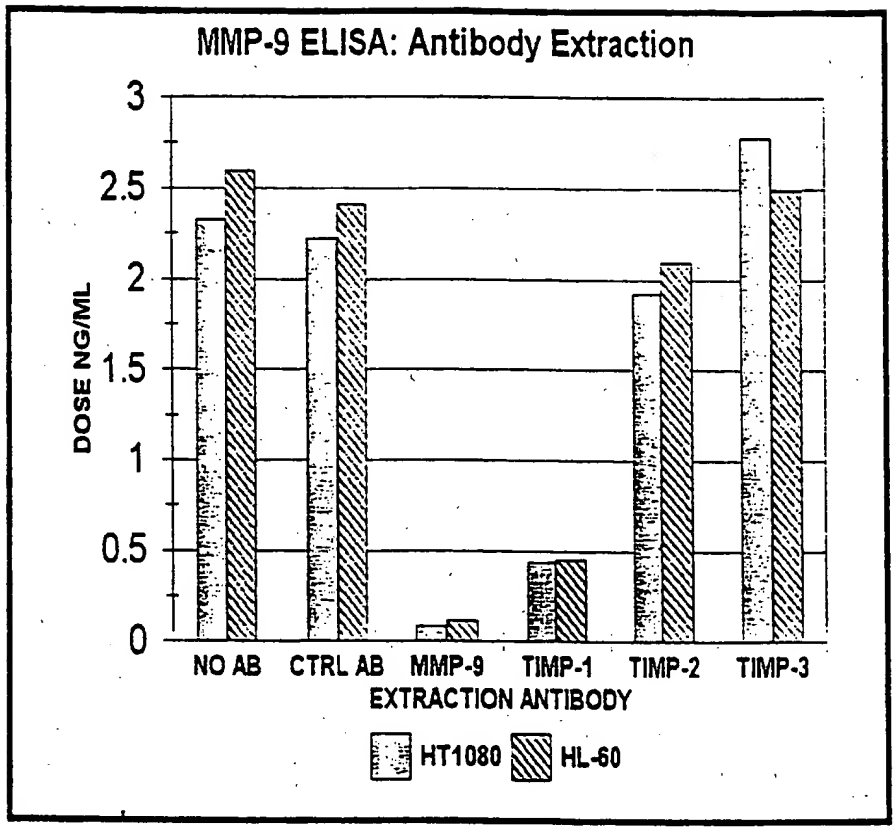

MMP-9 ELISA: Antibody Extraction
DOSE NG/ML
3
2.5
2
1.5
1
0.5
0
NO AB
CTRL AB
MMP-9
TIMP-1
TIMP-2
TIMP-3
EXTRACTION ANTIBODY
HT1080
HL-60

Figure 3.

Figure 4.

Figure 4A.

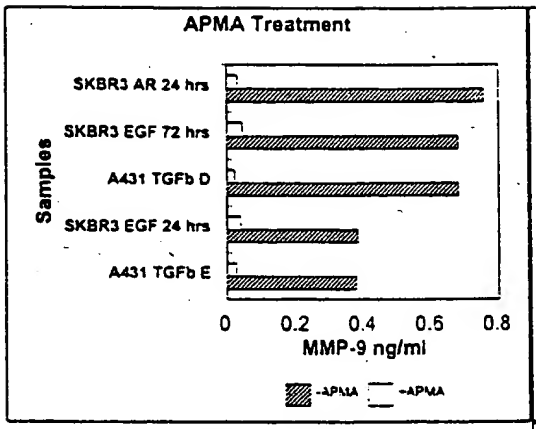
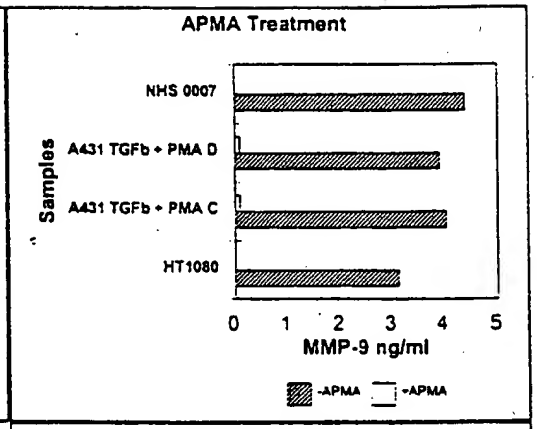


Figure 4B.



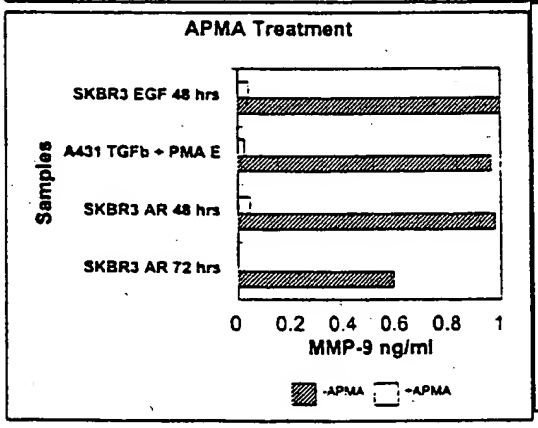


Figure 4C.

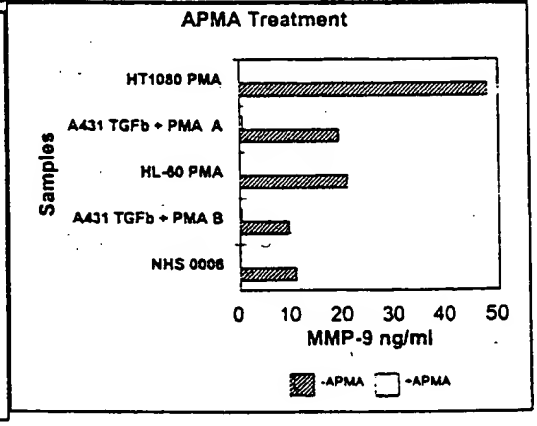


Figure 4D.

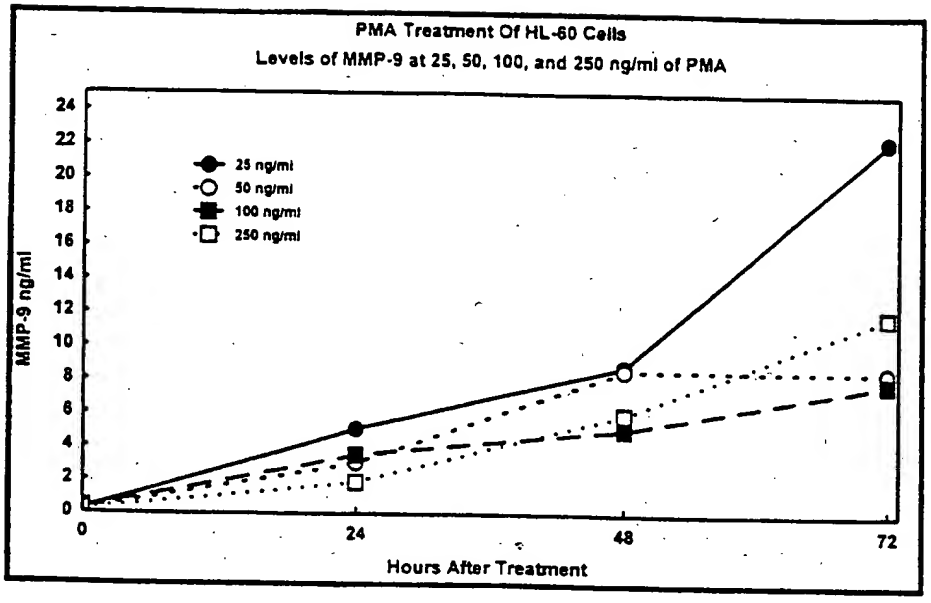


Figure 5.

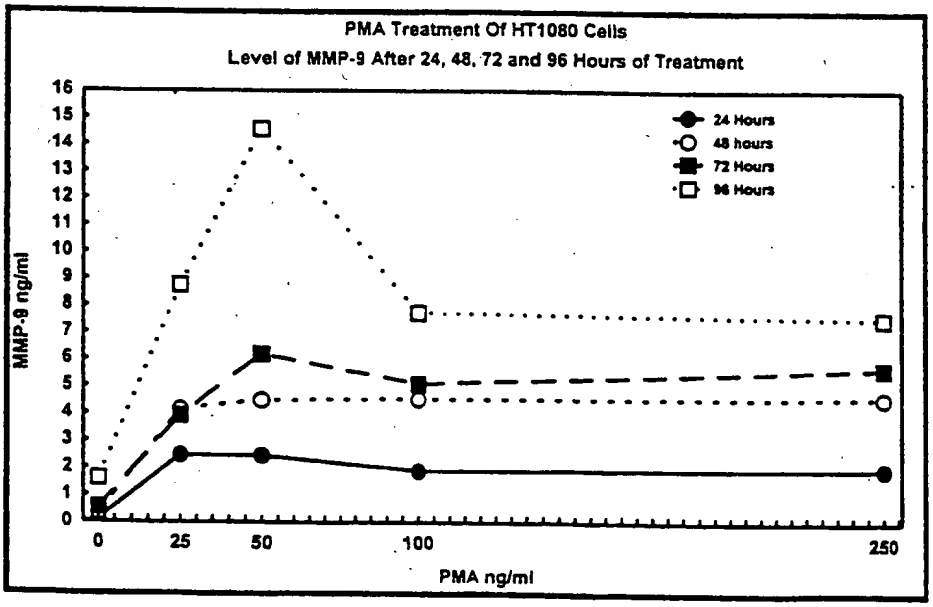


Figure 6.

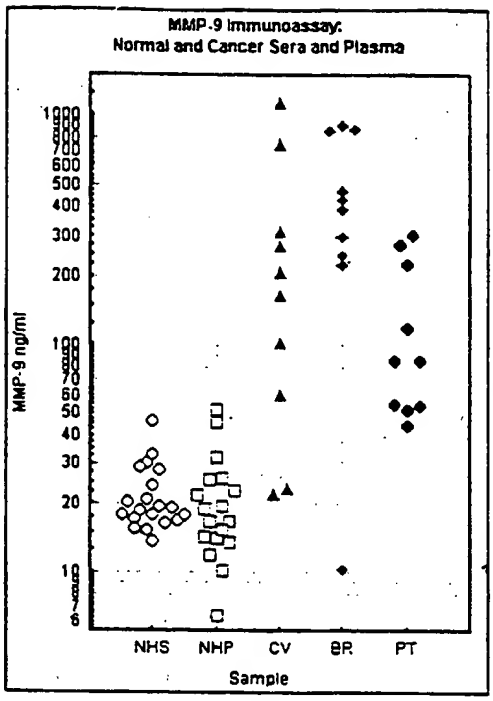


Figure 7. A.

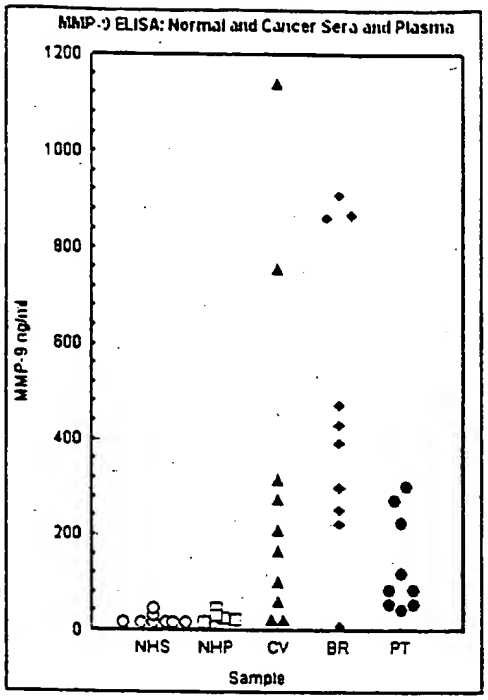


Figure 7. B.

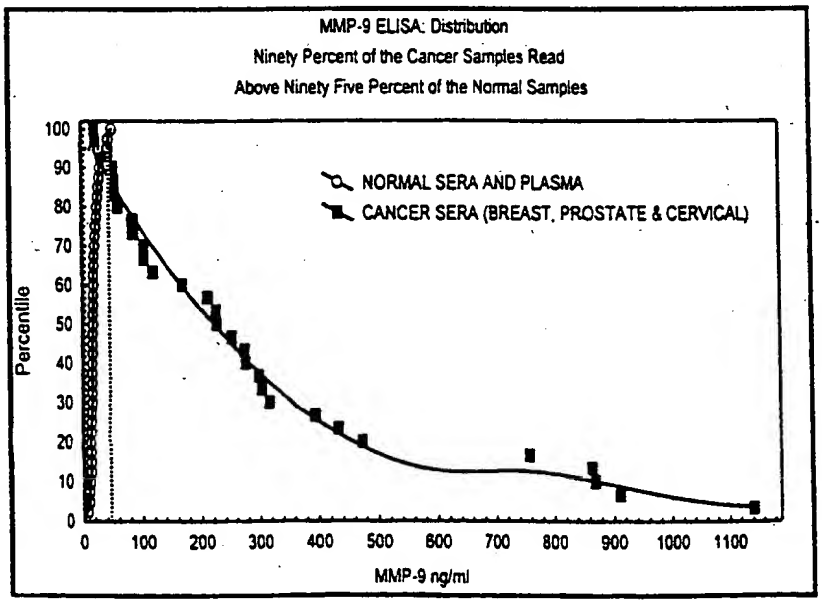


Figure 8.

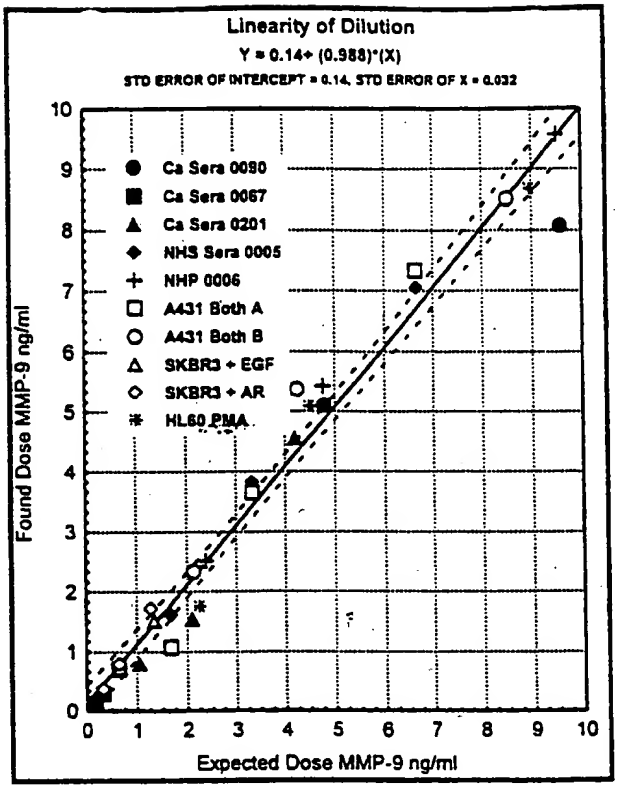


Figure 9.

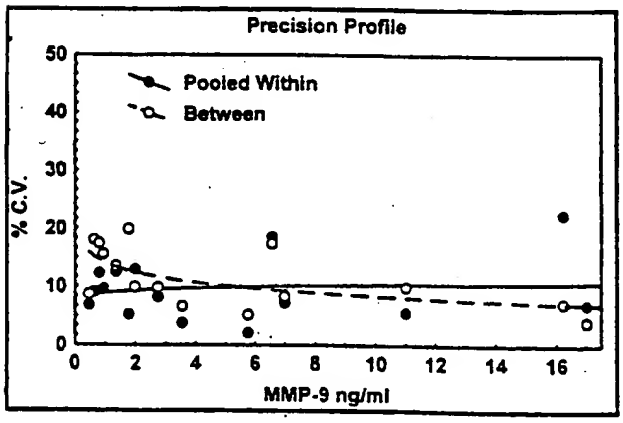


Figure 10.

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US99/23443

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/53,33/567,33/574
US CL : 435/7.1,7.2,7.23;436,503

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.1,7.2,7.23;436,503

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
STN, WEST

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZUCKER et al. Secretion of Gelatinases and Tissue Inhibitors of Metalloproteinases by Human Lung Cancer Cell Lines and Revertant Cell Lines: Not an Invariant Correlation with Metastasis, Int. J. Cancer, May 1992, Vol. 52 pages 366-371, especially page 367.	1-7
X	WO 97/38314 A1 (BRITISH BIOTECH PHARMACEUTICALS LIMITED) 16 October 1997, pages 1-21, especially pages 9,15,16, and 18.	1-10
X	WO 97/00449 A1 (ABERDEEN UNIVERSITY) 03 January 1997, pages 1-16, especially pages 9,10 and 15.	1-10
X	US 5,324,634 A (ZUCKER, S.) 28 June 1994, column 10, lines 59-68 and column 11, lines 1-34.	1-10
X	US 5,641,636 A (STRAUSS, J.F. III) 24 June 1997, column 5 lines 11-16; column 7, lines 55-67; column 10, lines 45-67.	1-10
X	ZUCKER et al. Mr 92,000 Type IV Collagenase Is Increased in Plasma of Patients with Colon Cancer and Breast Cancer. Cancer Research. January 1993, Vol. 53, pages 140-146, especially pages 140 and 143.	1-10
Y	US 5,674,754 A (AHRENS et al.) 07 October 1997, columns 8 and 9.	1-10
Y	ZUCKER et al. Comparison of Techniques for Measurement of Gelatinases/Type IV Collagenases: Enzyme-Linked Immunoassays Versus Substrate Degradation Assays. Clin. Exp. Metastasis. January 1994, Vol. 12, pages 13-23, especially page 15.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 December 1999 (01.12.1999)	31 JAN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Gary Nickol Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US99/23443

Continuation of B. FIELDS SEARCHED Item 3: Derwent, Medline, CAPLUS, BIOSIS,

terms: ELISA, MMP-9, sandwich, immunoassay, matrix metalloproteinase-9, cancer